Original article

# Spatzle4 gene of silkworm, *Bombyx mori*: identification, immune response, and the effect of RNA interference on the antimicrobial peptides' expression in the integument

Xinyi Huang [a], Ying Xu [a], Yang Zhang [a], Ji Liu [a], Shao Luo [a], Jinmei Wu [a,b,*]

[a] *College of Biotechnology, Jiangsu University of Science and Technology, Zhenjiang 212018, China*
[b] *The Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang 212018, China*

A B S T R A C T

Insects, including silkworms, can protect themselves from exotic invasions by innate immune responses. In the immune response of Drosophila, the activation of Toll receptors is strictly dependent on the product of Spatzle. In this study, we cloned the 1567 bp BmSpz4 cDNA which contained a complete 1386 bp open reading frame, encoding 461 amino acids, out of which the forgoing 19 residues were signal peptide. The result of the cDNA sequencing showed that we found a longer transcript than the one included in large scale full-Length cDNA sequencing data by Yoshitaka Suetsugu et al. in 2013. Several spliced variants of BmSpz4 have been found, based on our preliminary results. It was shown by the RT-PCR that BmSpz4 was expressed highest in the head, lower in the integument and testis. The expression of BmSpz4 in the integument of silkworm was upregulated by formalin-inactivated Gram-positive bacteria and fungi but not by Gram-negative bacteria, when compared to the control group of PBS injection. This phenomenon was the same as the one found in the Toll signaling pathway of Drosophila. In addition, the result of double-stranded RNA interference of BmSpz4 also demonstrated that it had a corresponding interference effect on the expression of the integument antimicrobial peptides induced by bacillus and yeast. Thus, it may be concluded that BmSpz4 plays an important role in the innate immunity against microbe infection in the integument of silkworm, *Bombyx mori*.

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## 1. Introduction

Insects are the largest and most diverse group of animals on the planet, with high adaptability and defense ability. Owing to some unique internal factors, the insects are of their ability to adapt to a variety of environments to survive and develop in the biological evolution. Like other invertebrates, insects, including silkworm, have no B or T lymphocytes, no immunoglobulin and its complement components, and not to mention the efficient, specific immune system that the higher animals possess, but they are able to protect themselves against exotic invasions by amounting various immune defense responses, known as innate immune responses. The innate immunity of insect includes physical defense, cellular immunity, humoral immunity. Insect physical defense mechanism is composed of many physiological barriers. The first line of defense against foreign infections of insects is an obstacle formed by their body structure, such as hard exoskeletons, trachea, and peritrophic matrix of midgut. The protection of the internal tissue of insects relies mainly on rapid coagulation (Theopolda et al., 2002) and melanization (Soderhall and Cerenius, 1998). Under these physical lines of defense, there are various epithelial cells immersed in insect haemolymph, which act as a functional physical defense to produce a strong immune response to microorganisms (Tingvall et al., 2001). Insect cellular immune signaling pathways contain extracellular signal pathways and intracellular signal pathways. The humoral immune system is mainly based on antimicrobial peptides, lectin, antiviral factors, lysozyme and protease inhibitors and other factors to establish a complete and open defense system with multifunctional blood cells (Jiang, 2006). The JAK/STAT, Toll, Imd, and RNAi pathways are the major signaling pathways associated with insect innate

immunity (Liu et al., 2015). The species variety and population of Lepidoptera are surpassed only by Coleoptera. In addition to silkworms and tussahs, there are many important crop pests such as cotton bollworm, oriental tobacco budworm, corn borer, Carolina sphinx moth, fall armyworm (Wang, 2015), and many more. The humoral immune factors in Lepidoptera insects, in addition to the presence of lectin, prophenoloxidase (Chai et al., 2014), and congenital immune factor in the insect body itself, the induction of microbes generates an acquired immune factor – antimicrobial peptides.

Insect antimicrobial peptides, a key part of the insect immune system, synthesized mainly by fat body, its function resembles the mammalian liver (Wang and Lai, 2010). The biological functions of antimicrobial peptides include antibacterial, antifungal, antiparasitic, antitumor, antiviral activities and so on. As an important anti-infective component of the innate immune system, antimicrobial peptides are widely expressed from insects, plants, to more advanced animals with complex immune systems (Mulder et al., 2013). The mechanism of action of antimicrobial peptides is very complex, including destroying cell membranes, affecting cytoplasmic components, interfering metabolism (Zhao et al., 2010) and so on. Antibacterial peptides have not only a strong antibacterial effect on Gram-positive bacteria and Gram-negative bacteria but a killing effect on certain fungi, viruses, cancer cells (Lamberty et al., 1999, Noorwal et al., 1994). In the early 1980s, Steiner and Salsted et al. isolated the insect antibacterial peptide Cecropin and Defensin for the first time, since then this type of antibacterial peptide began to get attention and its genetic immunity, host defense system, membrane protein interaction, protein modification and secretion and more had been studied. To date, more than 600 endogenous antibacterial active peptides have been found in many organisms including insects, birds, animals, plants and prokaryotes (Hancock, 2001). Among them, about 200 kinds of insect antimicrobial peptides were found (Toke, 2005). In addition, seven families of antimicrobial peptides that are inducible to microbe infection have been found from Drosophila so far (Hoffmann, 2003).

In 1980, Biologist Nüsslein-Volhard found a mutant gene that could lead to changes in the embryonic development of Drosophila, and the concept of "Toll" emerged for the first time. Toll protein, the necessary constituent protein to regulate the development of dorso-ventral axis during embryonic development, was first found in Drosophila and was involved in the formation of dorso-ventral pattern during Drosophila development (Anderson et al., 1985). At the beginning of this century, great progress has been made on the mechanism of the Drosophila immune system in recognizing pathogens, and two signaling pathways of Toll and Imd were identified by using genetic and molecular biological methodology (Hultmark, 2003, Hoffmann, 2003, Levitin and Whiteway, 2008, Ferrandon et al., 2007). Fungal or Gram-positive bacterial infections can activate the Toll signaling pathway of Drosophila. In contrast, there is growing evidence that the imd signaling pathway is primarily involved in the process of Drosophila resisting to Gram-negative bacteria and some Gram-positive bacterial infections (Leclerc and Reichhart, 2004, Tanji and Ip, 2005). In the Toll pathway, the soluble peptidoglycan (PGN) recognition protein PGRP-SA (Michel et al., 2001) and GNBP1 (initially thought to be a G-bacteria binding protein) mediate its activation (Gobert et al., 2003). They recognize the pathogen and transmit the signal to a proteolytic cascade process, leading to the cleavage of the cytokine-like protein ProSpaetzle (Spz). After Spz binds and activates the Toll receptor (Lemaitre et al., 1996), MyD88 associates with Pelle and Tube, in an heterotrimeric complex. Spatzle protein is an extracellular cytokine-like protein (Hoffmann and Reichhart, 2002). Its precursor is a non-active dimer ProSpatzle secreted by the cell, containing a pro-domain (25 kDa) and a cystine knot stuc-

ture (C-106) (14 kDa). A trypsin-like endonuclease Easter splices ProSpatzle, releases pro-damain, exposes the C-106 domain (Yu and Hou, 2010, Morisato and Anderson, 1994) binding to the extracellular domain on the two Toll molecules and activating the Toll signal pathway(Weber et al., 2007, Roh et al., 2009). It's shown through the sequence alignment that Spatzles of silkworm and Drosophila have a similarity of 54% in amino acid sequence. These two kinds of sequences make up one branch of the phylogenetic trees with the SPZ of Egyptian mosquito and Anopheles anthropophagus. Cheng (2008) suggested that there are six members in the Spatzle family in the silkworm genome, and the BmSpatzle1 gene is the first member in this family being cloned and studied on its functions.

Alternative splicing is prevalent in higher eukaryotes and is an important source leading to the complexity of higher eukaryotes transcripts. With the completion of human genome sequencing and gene annotations, it turns out that the predicted number of genes is far less than expected, whereas alternative splicing produces more transcripts. The spliced variants often exercise different biological functions, play essential role in the complexity of human biological functions (Matlin et al., 2005).

In this study, the full length cDNA of BmSpz4 gene was cloned and its sequence features were analyzed by means of bioinformatics. BmSpz4 expression profile, microbial induced expression and the effect of RNA interference on the antimicrobial peptides' expression were also carried out. The results showed that BmSpz4 cloned in this study contains a complete 1386 bp open reading frame, encoding 461 amino acids, out of which the N-terminal 19 residues were signal peptide. It expresses highest in the head while lower in the epidermis and testis. The immune response experiment and RNAi results clearly showed that BmSpz4 plays an important role against microbe infection in the integument of the silkworm.

## 2. Materials and methods

### 2.1. Silkworm varieties used in the experiments

p50 was used for BmSpz4 gene cloning, and the silkworm variety Suju × Minghu was used for the rest of the experiment. Silkworm eggs were incubated under the temperature of 25 °C and relative humidity of 80%. Silkworm larvae were fed with mulberry leaves. Escherichia coli, Saccharomyces cerevisiae and Bacillus subtilis were provided by our laboratory.

### 2.2. Cloning Spz4 cDNA from silkworm, Bombyx mori

The primers BmSpz4-ORF-F/BmSpz4-ORF-R (Table 1) were designed according to the predicted BmSpatzle4 sequence (XM_012691449.2) from NCBI. In order to clone the complete ORF of the gene, RNA was extracted from epidermal tissue of fifth instar larvae Bombyx mori, then cDNA was synthesized by reverse transcription with RNA as a template. The complete open reading frame was cloned and sequenced. The thermal cycling conditions were 94 °C 4 min, 30 cycles of 94 °C, 40 s; 55 °C, 30 s; and 72 °C, 60 s, followed by 10 min incubation at 72 °C. The reaction product was cloned into pMD18-T vector (TakaRa) and confirmed by DNA sequence analysis. And the complete ORF was cloned and sequenced eventually.

### 2.3. Bioinformatics analysis

Protein domains were predicted by the use of SMART program (http://smart.embl-heidelberg.de/). Spz protein sequences from other species in the database were searched and analyzed via the

**Table 1**
Summary of primers used in this study.

| Primers | Sequence(5′—3′) |
|---|---|
| *For ORF cloning* | |
| BmSpz4-ORF-F | GTTCTGTGGGTGCATGTTGC |
| BmSpz4-ORF-R | GAATCAGTCTTGAAGGCAGGA |
| *For genes expression* | |
| BmSpz4-F - | AGACGAGAAGAATACAACCGAT |
| BmSpz4-R | TCATAAGCGGACATGTACCAG |
| *For dsRNA templates amplification* | |
| DsRNA-BmSpz4-T7-F | **GGATCCTAATACGACTCACTATAGG** |
| | AGACGAGAAGAATACAACCGAT |
| DsRNA-BmSpz4-R | TCATAAGCGGACATGTACCAG |
| DsRNA-BmSpz4-F | AGACGAGAAGAATACAACCGAT |
| DsRNA-BmSpz4-T7-R | **GGATCCTAATACGACTCACTATAGG** |
| | TCATAAGCGGACATGTACCAG |
| *Antimicrobial peptide* | |
| BmAttacin-F | GGCTCGCTCTAGACAACGTA |
| BmAttacin-R | ACATTGGGCTCCCACGAAG |
| BmGloverin-F | TACTCGATCAGCGGGCAATC |
| BmGloverin-R | GACCATACCACCGGCAGAAA |
| BmMoricin-F | GCAAAAACAGTAAACCGCGCA |
| BmMoricin-R | CACAGTTGTCGTTGCAATCAAT |
| *Housekeeping gene* | |
| eTIF4A-F | TGGTGGGCACTCCAGGTCGT |
| eTIF4A-R | AGCCAATCCACCTTGCGACGG |

BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences of their Spaetzle domains were also analyzed independently by Clustal Omega Multiple Sequence Alignment (http://www.ebi.ac.uk/Tools/msa/clustalo/). ExPASy(http://au.expasy.org/tools/pi_tool.html)was used to predict the molecular weight and isoelectric point of the protein, and ProtScale (http://web.expasy.org/protscale/)predicting protein hydrophobicity. A neighbor-joining phylogenic tree was constructed by using the deduced amino acid sequences of Spzs through employing MEGA 6.0 software.

### 2.4. Tissue expression of BmSpz4

To examine mRNA levels of BmSpz4 in different tissues, total RNA samples were isolated from dissected fat body, midgut, silk gland, head, integument, ovary, and testis of the fifth instar silkworm larvae as previously described. By Semi-quantitative RT-PCR analysis, BmSpz4 gene expression was analyzed in different tissues on the third day of the fifth instar larvae.

To examine mRNA levels of BmSpz4 in different tissues, total RNA samples were isolated from dissected fat body, midgut, silk gland, head, integument, ovary, and testis of the day-3 fifth instar silkworm larvae. Primers BmSpz4-F/BmSpz4-R (Table 1) were used for BmSpz4 cDNA amplification by RT-PCR. Primers specific for *Bombyx mori* Eukaryotic Translation Initiation Factor 4A (eTIF4A, Accession Number: DQ443290.1) (Table 1) were used as an internal control.

The cDNA fragments were amplified at 94 °C for 3 min, 25 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. The PCR products were separated by gel electrophoresis and stained with ethidium bromide (EB).

### 2.5. BmSpz4 induction expression by microbe injection

To test the hypothesis that BmSpz4 did participate in immune responses, the levels of BmSpz4 mRNA was compared in integument after injecting to the silkworm larvae with PBS and different microorganisms.

Fifth-instar day-3 silkworm larvae were injected with phosphate buffer (PBS) (10 µl), Bacillus subtilis ($1.75 \times 10^5$ cells/ml, 10 µl), Escherichia coli ($1.96 \times 10^8$ cells/ml, 10 µl), Saccharomyces

cerevisiae ($1.55 \times 10^8$ cells/ml, 10 µl). The bacteria above have been formalin (6% formalin 25 °C for 30 min) treated or inactivated. Integument tissues were dissected at 24 h after injection for total RNA isolation. Then semi-quantitative RT-PCR was carried out as mentioned above.

### 2.6. Synthesis of double-stranded RNA for RNA interference experiments

The DNA templates of BmSpz4 dsRNA (designated as dsBmSpz4) were prepared via PCR using the primer pairs DsRNA-BmSpz4-T7-F/DsRNA-BmSpz4-R and DsRNA-BmSpz4-F/DsRNA-BmSpz4-T7-R (Table 1). Products with a T7 promoter were confirmed via sequencing. Subsequently, following the manufacturer's protocol, the products were used as templates for the sense and antisense RNA strands, subjected to transcription in vitro, and purified by the use of T7 RiboMAX™ Express RNAi System (Promega, USA). The DsBmSpz4 was 684 bp in length.

Day 3, 5th instar silkworms were divided into six groups including three experimental groups and three control groups. Experimental groups were injected with 10 µl (1 µg/µL) of BmSpz4-specific DsRNA(DsBmSpz4) and 6 h later with Bacillus subtilis ($1.75 \times 10^5$ cells/ml, 10 µl), Escherichia coli ($1.96 \times 10^8$ cells/ml, 10 µl), Saccharomyces cerevisiae ($1.55 \times 10^8$ cells/ml, 10 µl). In contrast, control groups were injected with 10 µl PBS and 6 h later with Bacillus subtilis ($1.75 \times 10^5$ cells/ml, 10 µl), Escherichia coli ($1.96 \times 10^8$ cells/ml, 10 µl), Saccharomyces cerevisiae ($1.55 \times 10^8$ cells/ml, 10 µl). mRNA was extracted from the integument 6 h after the injection of microorganisms. RT-PCR was used to observe the expression of antimicrobial peptides.

## 3. Results

### 3.1. Cloning and sequencing of BmSpz4 gene

The results of RT-PCR gel electrophoresis of cloned BmSpz4 cDNA (Fig. 1) showed that the product size of RT-PCR is between 1000 bp
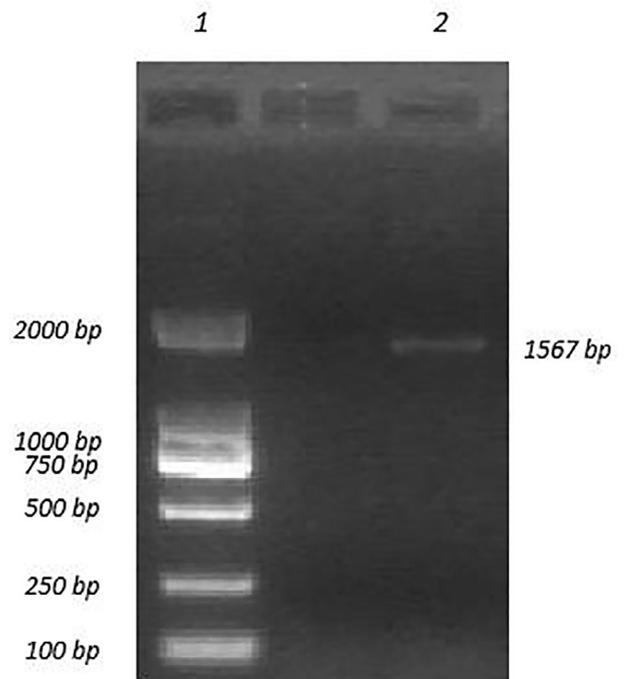


**Fig. 1.** BmSpz4 cDNA amplified by PCR, as shown by gel electrophoresis. 1: DL2000 Marker; 2: PCR amplification product.

and 2000 bp, which was in accordance with the expected product size of 1567 bp. The sequence of the cloned product was submitted to GenBank, and the accession number obtained is MG196034. The ORF of BmSpz4 was composed of 1386 bp nucleotides (Fig. 2), encoding 461 amino acids. A conservative domain analysis using the SMART program indicates that BmSpz4 contains a spatzle domain. The family of proteins is the nerve growth factor-like ligand required to establish the dorsal-ventral pattern of the Drosophila embryo (Morisato and Anderson, 1994). BmSpz4 contains a signal peptide at its amino terminus, as shown in Fig. 2. Suetsugu et al. (2013) obtained a cDNA clone (AK381775.1) in 2013, which is 95 bp shorter in the 5′UTR region compared to the sequence



Fig. 2. cDNA sequence cloned in this experiment and the deduced amino acid sequences of the coding region of BmSpz4. The start codon is shown in the box; Star represents the stop codon; The conserved domains are shaded, and signal peptide at the amino terminal is colored dark grey and in bold font.

obtained in this experiment. The ORF of BGIBMGA008841-TA and JN628282 has a total length of 1278 bp, encoding 425 amino acids, with no signal peptide. The ORF of MG196034 was 108 bp longer than JN628282, and the 108 bp encodes 36 amino acids at the 5′end,

among which the first 19 amino acids were the signal peptide. As is shown in Fig. 3, there are several possible splicing variants of BmSpz4. The comparison of BmSpz4 in this experiment with JN628282 and AK381775.1 is shown in the figure.
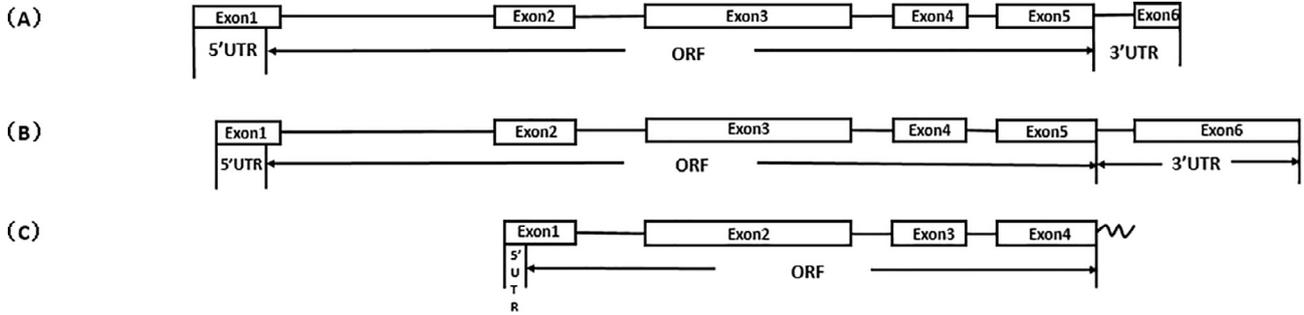


**Fig. 3.** Schematic diagram of corresponding exons of the alternative spliced variants of BmSpz4. The boxes represent exons and the lines represent introns. (A) MG196034; (B) AK381775.1; (C) JN628282.



**Fig. 4.** Multiple amino acid sequence alignment of the spaetzle proteins from different insects. AgSpz(Anopheles gambiae Spz:XP_317626.4), AdSpz(Apis dorsata Spz: XP_006612983.1), BmSpz4(*Bombyx mori* Spz4:XP_012546903.1), DmSpz4(Drosophila melanogaster Spz4: NP_609504.2), NvSpz(Nasonia vitripennis Spz: XP_008202261.2), TcSpz4(Tribolium castaneum Spz4: EFA09263.2).

```
BmSpz4    ----------------------S-------------IANIEINNLDQEIITLEAVIKQSI
AdSpz     --------------VTTSPTVTSSY---T-----VKEQNFSINEISEQIDRIDE----VV
NvSpz     --------------RSTTPVVSLAQ---I-----AKIQNLSINEISGQIDRFDA----DV
TcSpz4    ------------------------------------------------------------
AgSpz     S------TTTASSTGTTNPSVESKAEQ----------QHATTTSTPSTADKISP------V
DmSpz4    SVYEVVSPSVLSTTSTAKPSSAAGENIQIIDSPQLGLRNLSAEASPSQEPPTAT-----T


BmSpz4    ETNSIYPKSSTDIDAIDEK------------NTTDI--SNATSADFINTTDVITDKLEET
AdSpz     EEAGEVIE----VSE--SN----------------I--EETTTMNFPGTTELPD------
NvSpz     DELTEIPG----ILIEEGF----------------A--ADTTADSLDGIPTSSHQYLSTS
TcSpz4    -------EETT----------------------TVETATTDTTDT---------------
AgSpz     GGSSVVPTNKNS---------------------IEAADTEAIDMDALSSL------A-
DmSpz4    GKPTSVPKSTTPLPIRKRKPAETTTSAASPKVSSTISTPTTTTATLLIRRNV-TKYSPA-
                                                            :  :


BmSpz4    ---TESGK----------------DGWNPMNVD-----T-STLPP-----TLLFSEHERIK
AdSpz     ---ELDGSE--DLFATPKETT----------VQEEE-TSNSNEQQEFRPRPEYRPSK---
NvSpz     SERTDDGKISLEDYGSKFDQV----------QDAEV-SESQQQPQQFRPRPEYRPPEAVR
TcSpz4    ------------------------------------TNDNN------------NPT---
AgSpz     ---KLETN---ESLQATFASGDKD------VEETVVKISST--------------LKLAS
DmSpz4    ---SVTTP---VSFASLFSGTSSGLFSPERLRRPLTTTSSTSAPAS-S-PPAGGTPASSS
                                                       .


BmSpz4    IEKNENKINEKKDEVRVKPQHQE-GSRPAVIKLGGANACESTETLTAPFWANSTRGEVLA
AdSpz     -----PEVSTMEGQLYQDVAAKDQQEQ-PVLKLRGVNACPVKEEVVAPFWANNTRGEVLA
NvSpz     PEAQGPTGGSMEGQLFQDVAAKDKQ-E-PVLKVRGINACPVKEEVVAPFWANNTRGEVLA
TcSpz4    --ENDSGDNNPQTVLFQDMEDKNSHKVQVNYHMKGVNACPVKEEVVAPFWANNTRGEVLA
AgSpz     SSAPEKKTSNPEGQLFQDAAQKE--PPVV--NGRGVNACPVKEEVVAPFWANNTRGEVLA
DmSpz4    TGSASKSGLLREGQLFQDAMKQE--PVAVASNLRGVNACPVKDEVVAPFWANNTRGEVLA
                    :   :     .      ::          :     * ***   .:  :.******.*******


BmSpz4    LLNMHPFEQYIHMETCLHERKQMYCREGCRCEQQYRLHRLLAYDPRNECRGIFADWFRFP
AdSpz     LLNLYPFEQYVHWEKCTHENKQMYCRDGCRCEQQYRLHRLLAYDPNNECRGIFSDWFKFP
NvSpz     LLNLYPFEQYVHWEKCTNENKQMYCRDGCRCEQQYRLHRLLAYDPNNECRGIFSDWFKFP
TcSpz4    LLNMYPFEQYVHWEKCTHEHRQMYCRDGCRCEQQYRLHRLLAYDPNNECRGIFSDWFRFP
AgSpz     LLNLYPFEQYVHWEKCTHELKQMYCREGCRCEQQYRLHRLLAYDPHNECRGIFSDWFRFP
DmSpz4    LLNLYPFEQYVHWEKCTHEFKQMFCRDGCRCEQQYRLHRLLAYDPHNECRGIFSDWFRFP
          ***::*****:* *.* :* :**:**:*****************.*******:***:**


BmSpz4    TCCVCKCYDVP-VEFRA--RSPRILHPQ----------YDEKVKRVIFEDVARDWYMSAY
AdSpz     SCCVCRCYDLP-LEFRVTSRSPRTQKQ--RIKVQSPRY--------------------T---
NvSpz     SCCICRCYDLP-VEFRVTSRSPRLVHQRQRLKVRAPRA-------------------PPRQ
TcSpz4    SCCVCKCYSIPPAEFRVTSRSPRAFDDG---------------------QVPPDWYRRKN
AgSpz     SCCICKCYDMP-FDFRVTSRSPRSLT------KESIELVEDELQNAIYEHAADEWYRPKE
DmSpz4    SSCICKCYNIP-MEFRATSRSPRSDSRFDAPKSDPIEAAEAEVQRAIYEHATEEWYRPRD
          :.*:*:*:**.:*   :**.    ****


BmSpz4    DDEDFL----
AdSpz     ----------
NvSpz     RH-H------
TcSpz4    EA-IFGGDHG
AgSpz     HD-VE-----
DmSpz4    EF-DFLED--
```

**Fig. 4** (*continued*)

## 3.2. Phylogenetic analysis

ExPASy predicted that the molecular weight of BmSpz4 protein was 53.19 kD and the isoelectric point was 5.56. It was showed by the hydrophobicity analysis of the amino acid sequence of ProtScale that the maximum hydrophobicity of BmSpatzle4 protein was 3.333, the minimum was −3.011, and the hydrophobic and hydrophilic regions of the protein sequence were staggered.

Through the NCBI Blastp (http://blast.ncbi.nlm.nih.gov/Blast.cgi) we conducted protein sequence alignment online, the amino acid sequence of BmSpz4 and those of Anopheles gambiae, Apis dorsata, Drosophila melanogaster, Nasonia vitripennis, Tribolium castaneum SPZ have closer relationship than those of mammalian SPZs. By sequence alignment and evolution analysis, it is shown that the newly cloned spatzle gene of silkworm is the closest to the spatzle 4 gene in Drosophila melanogaster, so it is named as BmSpz4 (Fig. 4).

According to the similarity of amino acid sequences, the phylogenetic tree of BmSpz4 was constructed with homologous proteins from other species, including Aedes aegypti, Anopheles gambiae, Apis dorsata, Danio rerio, Drosophila melanogaster, Harpegnathos saltator, Helicoverpa armigera, Homo sapiens, Mus musculus, Musca domestica, Nasonia vitripennis, rapae, Tribolium castaneum, Zootermopsis nevadensis (Fig. 5).

## 3.3. Tissue expression profile of BmSpz4

The result shows that BmSpz4 is expressed differently in tissues and is detected the highest expression level in head, followed by integument and testis. And there is no expression of this gene in the rest of the tissues (Fig. 6).

## 3.4. Microbe-induced expression analysis of BmSpz4

There was a significant increase of Bmspz4 24 h after the immune challenge of Bacillus subtilis and Saccharomyces cerevisia,
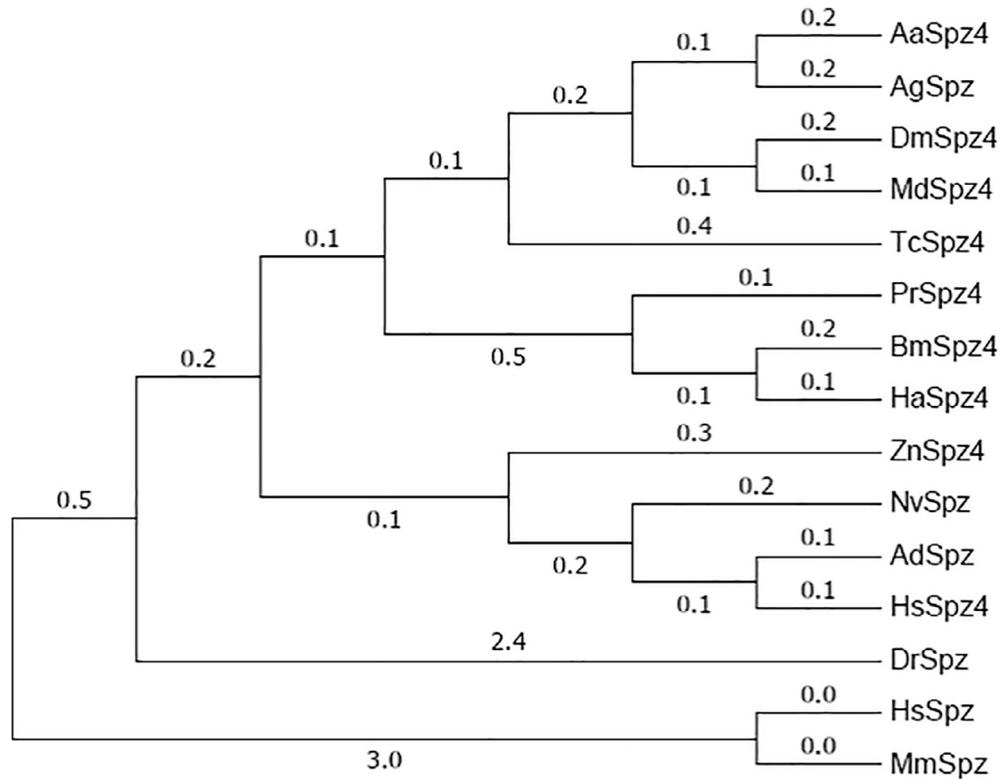


**Fig. 5.** Phylogenetic tree of various Spatzle proteins AaSpz4(Aedes aegypti Spz4: XP_021704087.1), AgSpz(Anopheles gambiae Spz:XP_317626.4), AdSpz(Apis dorsata Spz: XP_006612983.1), BmSpz4(*Bombyx mori* Spz4:XP_012546903.1), DrSpz(Danio rerio Spz: XP_696044.4), DmSpz4(Drosophila melanogaster Spz4: NP_609504.2), HsSpz4 (Harpegnathos saltator Spz4: XP_011136416.1), HaSpz4(Helicoverpa armigera: XP_021190976.1), HsSpz(Homo sapiens Spz: NP_005892.1), MmSpz(Mus musculus Spz: NP_034884.2), MdSpz4(Musca domestica Spz4: XP_005184799.1), NvSpz(Nasonia vitripennis Spz: XP_008202261.2), PrSpz4(Pieris rapae Spz4: XP_022116075.1), TcSpz4 (Tribolium castaneum Spz4: EFA09263.2), ZnSpz4(Zootermopsis nevadensis Spz4: XP_021920622.1).
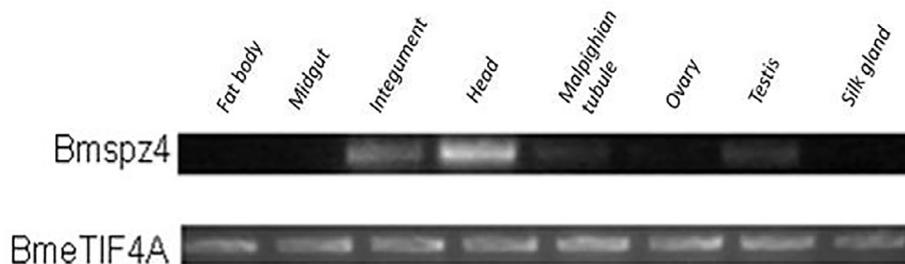


**Fig. 6.** Expression profile of BmSpz4 gene on day 3 of the 5th instar silkworm larva. The silkworm eTIF4A was used as an internal standard to normalize the templates.
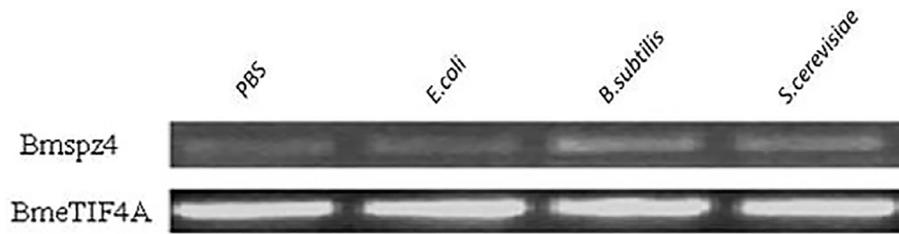
**Fig. 7.** Immune responses of BmSpz4 after microbial injection into the Day-3 fifth instar silkworm larvae. The silkworm eTIF4A was used as an internal standard to normalize the templates.
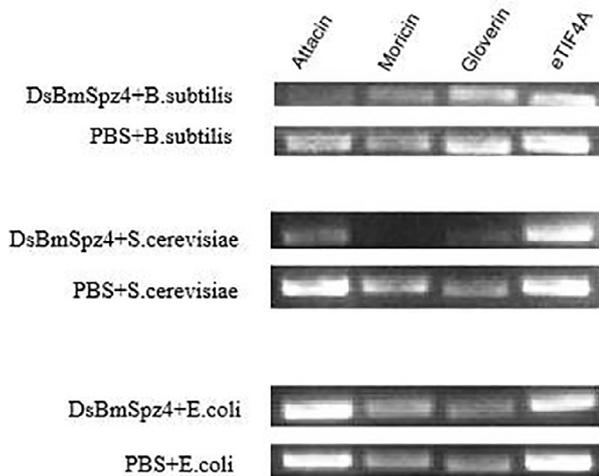


**Fig. 8.** Expression of antimicrobial peptides in the integument of *Bombyx mori* larvae after injection of dsBmSpz4 or PBS, and then challenged by microorganism as indicated.

and no obvious increase in the gene's transcription when injected with Escherichia coli (Fig. 7).

### 3.5. RNA interference

We investigated the effect of RNA interference of BmSpz4 gene on the expression of antimicrobial peptides in the integument of the silkworm, *Bombyx mori*. We injected the fifth instar day-3 silkworm larva with DsBmSpz4. The results showed that the expression of antimicrobial peptides in the body wall of the silkworm was significantly decreased in the experimental group of injecting DsBmSpz4 for RNA interference then challenged by microbes, compared to the control group in which no DsBmSpz4 but instead PBS was injected then challenged by microbes (Fig. 8). It can be seen from the results above that BmSpz4 gene in the integument of silkworm does play an important immune function.

### 4. Discussion

The existing BmSpz4-homologous sequences in the NCBI's GenBank datadase are the ones without the signal peptide, the corresponding predicted gene in the silkworm genomic database SilkDB also does not include a signal peptide in the deduced protein, but the results of our study showed that there did exist another BmSpz4 containing the signal peptide. So, there is a BmSpz4 protein with a signal peptide and also a spliced variant without signal peptide. When a signal peptide is cleaved and then the mature protein is released into the endoplasmic reticulum and is finally transported to extracellular space (Ye, 1999). The signal peptide is located at the N end of the secreted protein, playing a

leading role in the secretion of exocrine proteins (Izard et al., 1995, Yang et al., 2000). It can be seen that signal peptide plays an important part in protein transport. And what we gained from the cloning through this study is BmSpz4, in which the signal peptide contained. In this study, we cloned a novel BmSpz4 isoform with a N-terminal signal peptide, which may indicate its important role in the secretion of the protein to the extracellular space.

Judging from the preliminary results we conclude that there are several spliced variants of BmSpz4. Alternative splicing refers to a process, through which the precursor mRNA splices in different ways and produces different transcripts. Alternative splicing is an important means how the organisms achieve self-regulation. The analysis of alternative splicing events allows people to understand the complexity of organisms from the perspective of transcripts. Alternative splicing greatly enriches the abundance and complexity of proteins and species transcripts, plays an important role in the regulation of organogenesis, tissue differentiation, and environmental response of higher organisms. Alternative splicing play very important parts in the change of biological function of organisms, such as: The sex determination of the Drosophila is determined by alternative splicing (Lopez, 1998, Salz, 2011); the control of meiosis in yeast (Engebrecht et al., 1991). All of these is greatly relevant to alternative splicing. The existence of several spliced variants in BmSpz4 may be of great significance.

Cheng (Cheng, 2008) suggested that there exist six members in the Spatzle family among silkworm genome. BmSpatzle-1 gene is the first gene of the family to be cloned and studied on the relevant functions. After injecting different types of microorganisms into the body cavity of silkworm larvae, the expression level of BmSpz 1 was upregulated by Gram-positive bacteria, Gram-negative bacteria and fungi. Gram-negative bacteria and Gram-positive bacteria can induce the expression level of BmSpz-1 up to about 2 times, while fungi can make it increased by about 4 times (Wang et al., 2007). According to their analysis to the humoral immune factor BmSpz-1, it's shown that the Toll signaling pathway can be induced by Gram-negative bacteria, Gram-positive bacteria and fungi, which differs from the microbial immune induction results in Drosophila that can only be activated by Gram-positive bacteria and fungi, while the Imd signal pathway can be activated by Gram-negative bacteria and some Gram-positive bacteria (Michel et al., 2001).

Liu et al. (2016) in our lab suggested that BmSpatzle5 was involved in the Toll signal pathway in the silkworm, and the level of BmSpz5 expression was up-regulated by Gram-positive bacteria and fungi, but not by Gram-negative bacteria. In this study, it was shown that the expression of BmSpz4 was up-regulated by formalin-inactivated Gram-positive bacteria and fungi but not by Gram-negative bacteria after injecting different types of microorganisms to the body cavity of silkworm larvae. So, BmSpz4 and BmSpz5 are similar in the microbial induction expression, with the pattern in accord with the classical Drosophila Toll signaling pathway. In addition, it was also shown from the result of BmSpz4 double-stranded RNA interference that the expression of the

antimicrobial peptides induced by yeast and Bacillus is correspondingly interfered. Thus, as an important part of silkworm natural immunity, BmSpz4 in the integument may play a key role in the silkworm, *Bombyx mori*.

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